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(54) **MITOCHONDRIAL DNA DELETION BETWEEN ABOUT RESIDUES 12317-16254 FOR USE IN THE DETECTION OF CANCER**

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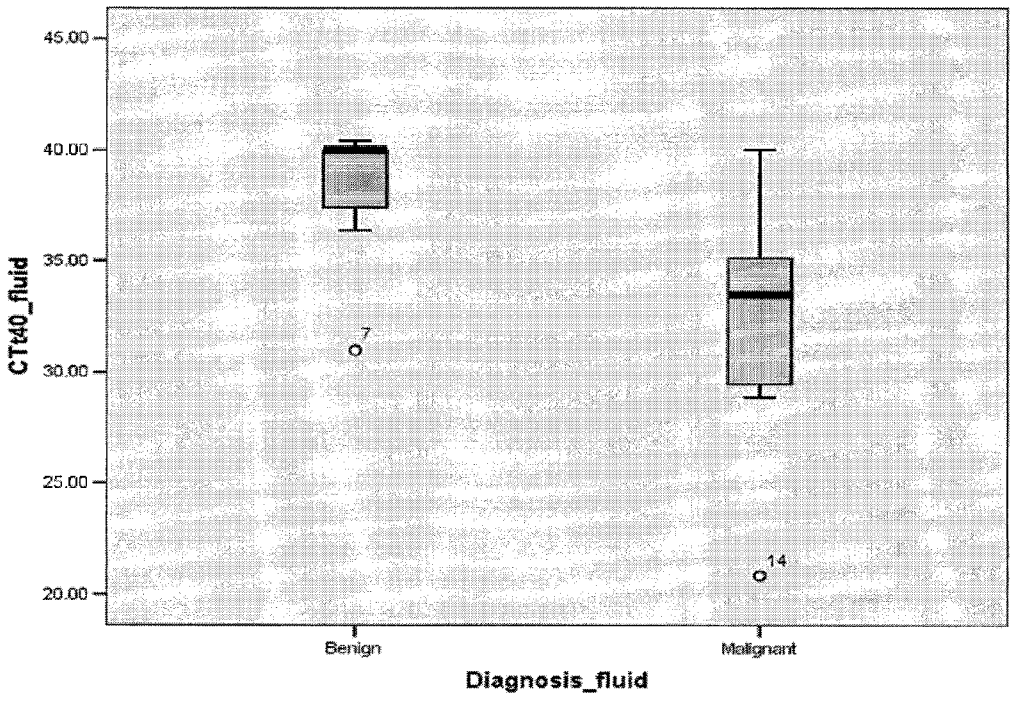
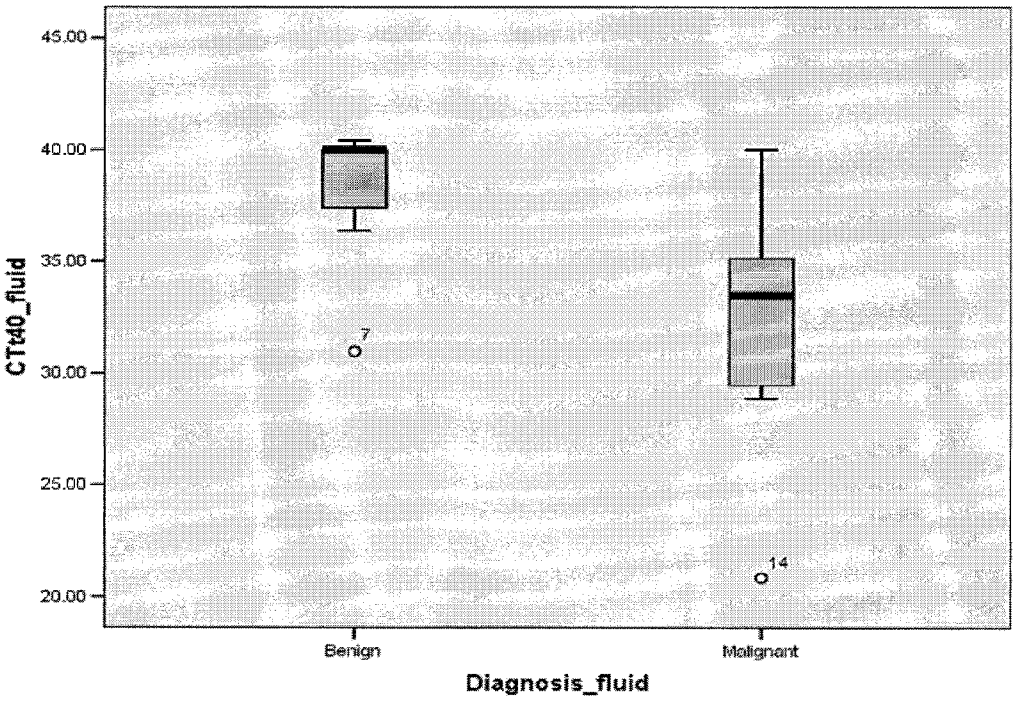
(57) **ABSTRACT**

The present invention relates to methods for predicting, diagnosing and monitoring cancer. The methods comprise obtaining biological samples, extracting mitochondrial DNA (mtDNA) from the samples, quantifying mitochondrial DNA mutation in the sample and comparing the level of mtDNA mutation with a reference value. The methods of the invention may also be effective in screening for new therapeutic agents and treatment regimes, and may also be useful for monitoring the response of a subject to a preventative or therapeutic treatment.




45.00
40.00
35.00
30.00
25.00
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CT40_fluid
7
14
Benign
Malignant
Diagnosis_fluid

Figure 1

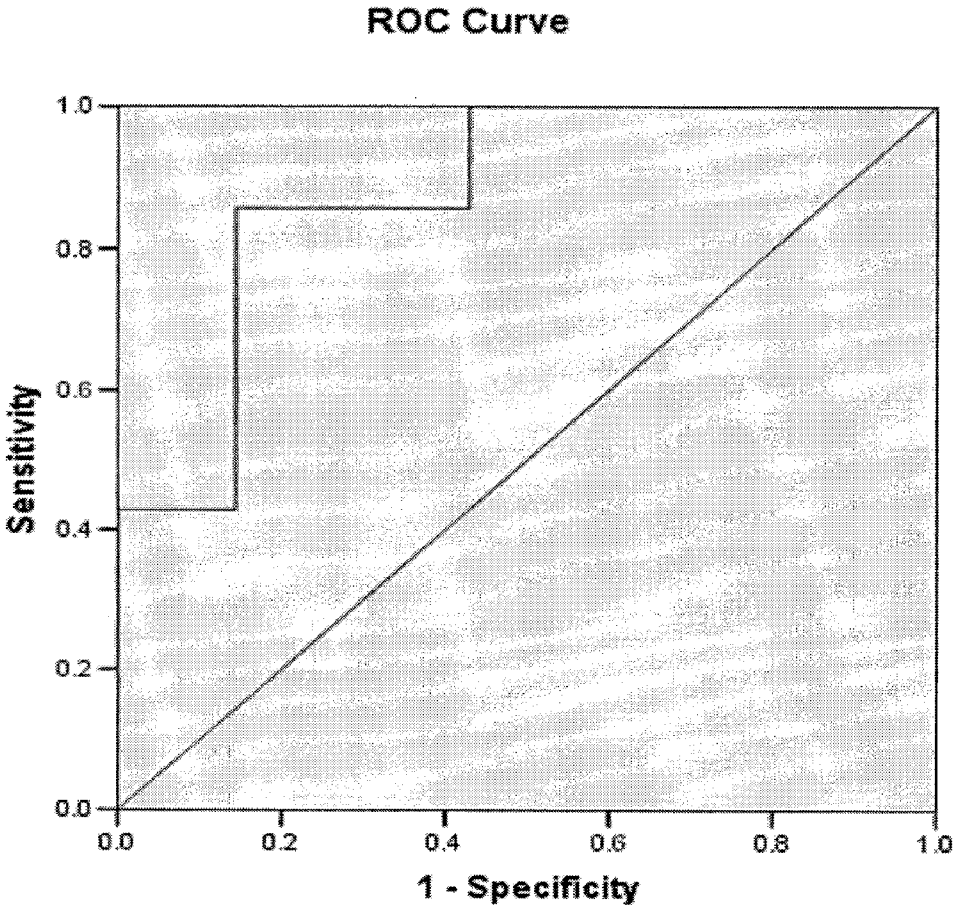


Figure 2

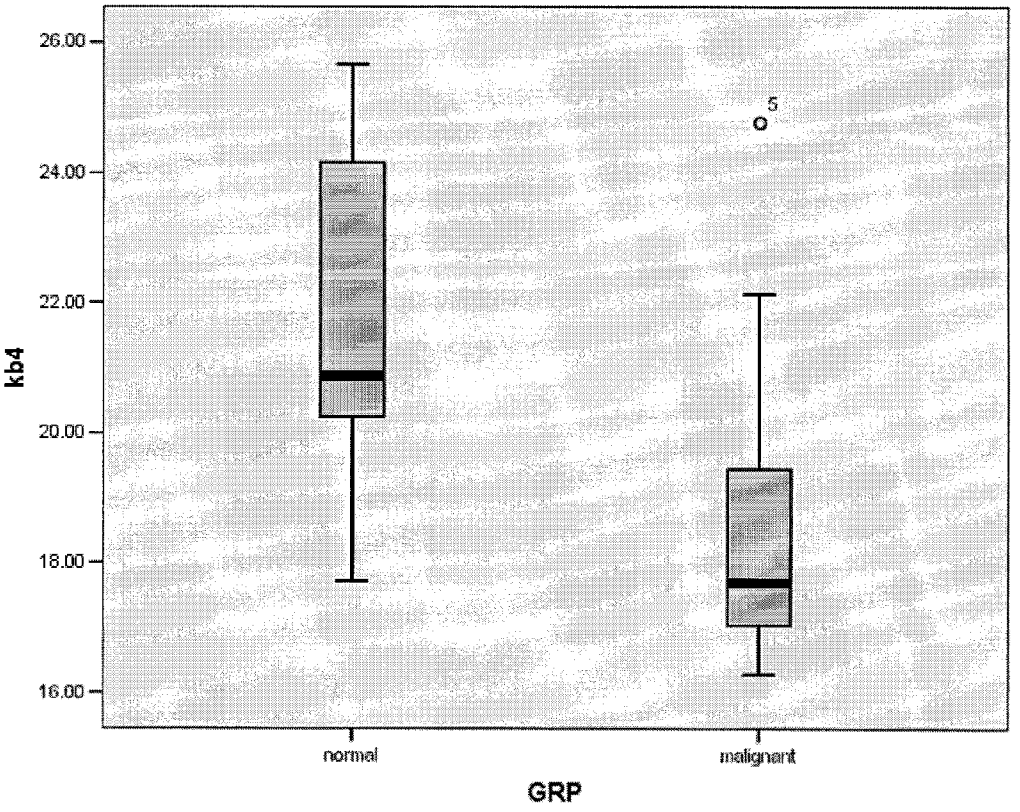


Figure 3

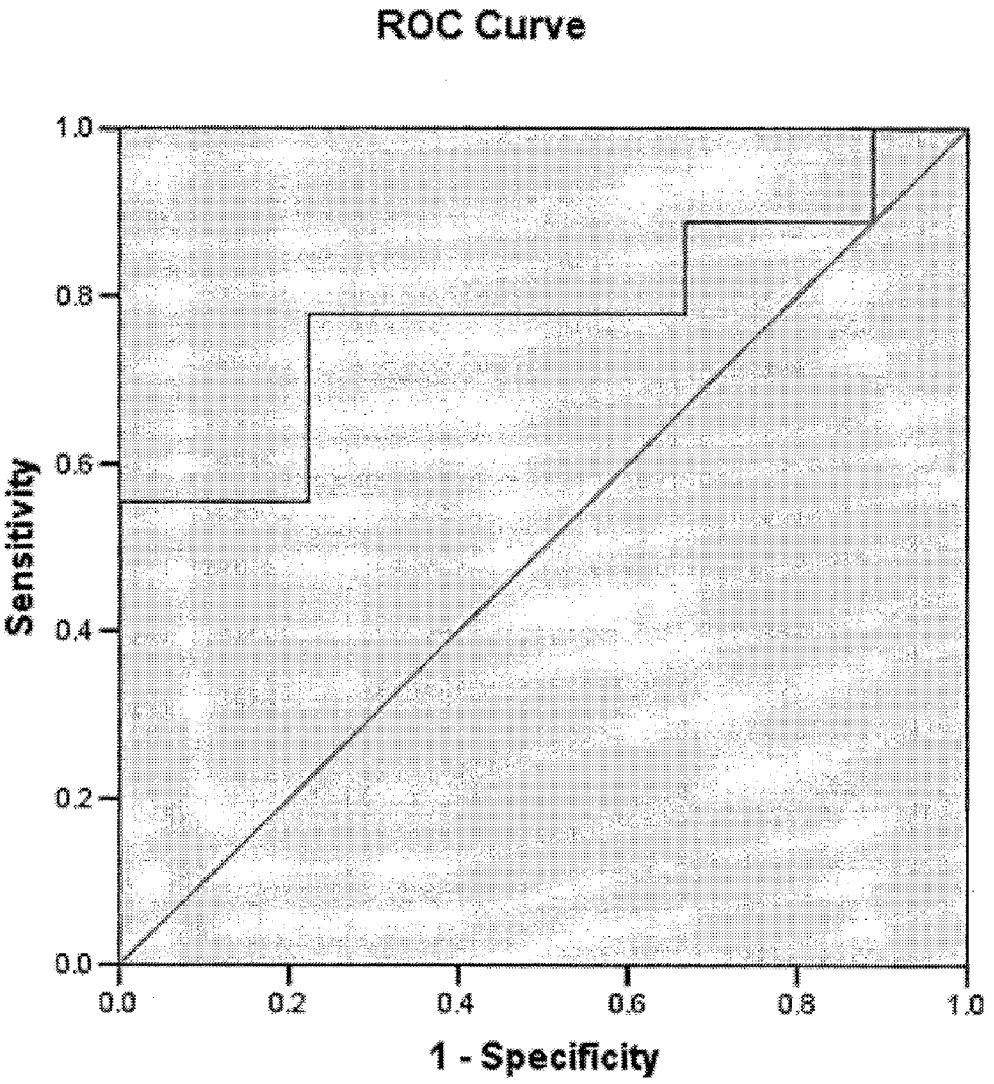
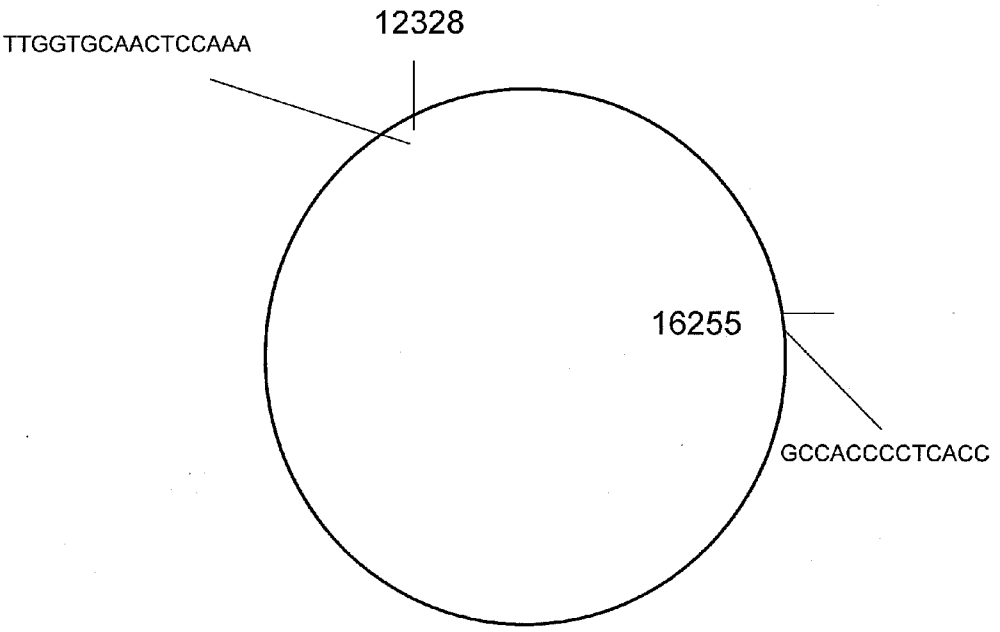


Figure 4

Agent Ref: 102222/00050
21826003.1

Primer Design for 4kb Deletion Detection



PRIMER SEQUENCE 5'-TTGGTGCAACTCCAAAGCCACCCCTCACC-3'

Figure 5

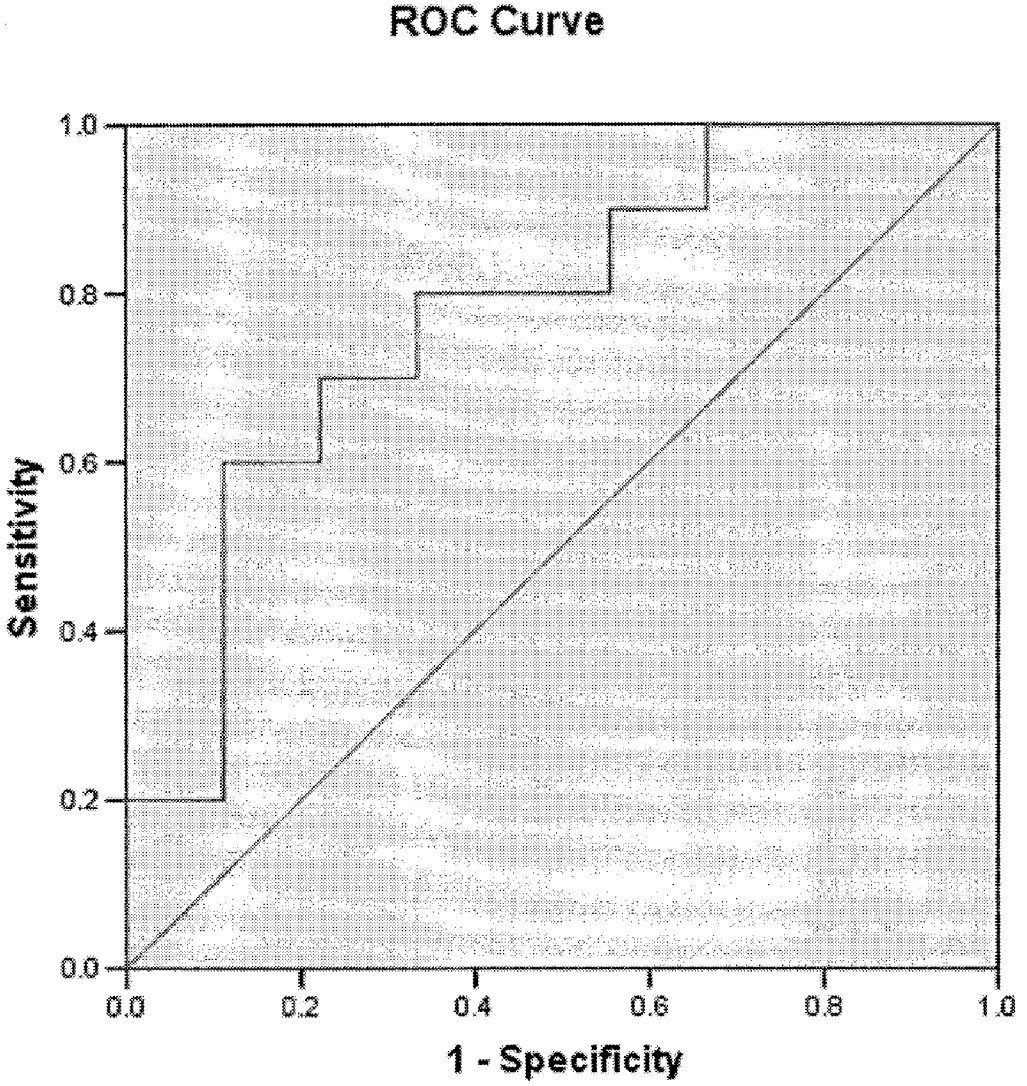


Figure 6

MITOCHONDRIAL DNA DELETION BETWEEN ABOUT RESIDUES 12317-16254 FOR USE IN THE DETECTION OF CANCER

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority from U.S. Application No. 61/002,637, filed Nov. 9, 2007, the entire contents of which are incorporated herein by reference.

FIELD OF THE INVENTION

[0002] The present invention pertains to the field of mitochondrial genomics. In particular it is related to the detection of human mitochondrial genome mutations and their utility as an indicators of cancer.

BACKGROUND OF THE INVENTION

Mitochondrial DNA as a Diagnostic Tool

[0003] Mitochondrial DNA (mtDNA) sequence dynamics are important diagnostic tools. Mutations in mtDNA are often preliminary indicators of developing disease, often associated with nuclear mutations, and act as biomarkers specifically related to: disease, such as but not limited to, tissue damage and cancer from smoking and exposure to second hand tobacco smoke (Lee et al., 1998; Wei, 1998); longevity, based on accumulation of mitochondrial genome mutations beginning around 20 years of age and increasing thereafter (von Wurmb, 1998); metastatic disease caused by mutation or exposure to carcinogens, mutagens, ultraviolet radiation (Birch-Machin, 2000); osteoarthritis; cardiovascular, Alzheimer, Parkinson disease (Shoffner et al., 1993; Sherratt et al., 1997; Zhang et al, 1998); age associated hearing loss (Seidman et al., 1997); optic nerve degeneration and cardiac dysrhythmia (Brown et al., 1997; Wallace et al., 1988); chronic progressive external exopthalmoplegia (Taniike et al., 1992); atherosclerosis (Bogliolo et al., 1999); papillary thyroid carcinomas and thyroid tumours (Yeh et al., 2000); as well as others (e.g. Naviaux, 1997; Chinnery and Turnbull, 1999).

[0004] Mutations at specific sites of the mitochondrial genome can be associated with certain diseases. For example, mutations at positions 4216, 4217 and 4917 are associated with Leber's Hereditary Optic Neuropathy (LHON) (Mitochondrial Research Society; Huoponen (2001); MitoMap). A mutation at 15452 was found in 5/5 patients to be associated with ubiquinol cytochrome c reductase (complex III) deficiency (Valnot et al. 1999).

[0005] Specifically, these mutations or alterations include point mutations (transitions, transversions), deletions (one base to thousands of bases), inversions, duplications, (one base to thousands of bases), recombinations and insertions (one base to thousands of bases). In addition, specific base pair alterations, deletions, or combinations thereof have been found to be associated with early onset of prostate, skin, and lung cancer, as well as aging (e.g. Polyak et al., 1998), premature aging, exposure to carcinogens (Lee et al., 1998), etc.

Prostate Cancer

[0006] Prostate cancer is a frequently diagnosed solid tumour that most likely originates in the prostate epithelium (Huang et al. 1999). In 1997, nearly 10 million American men were screened for prostate specific antigen (PSA), the presence of which suggests prostate cancer (Woodwell, 1999). Indeed, this indicates an even higher number of men screened by an initial digital rectal exam (DRE). In the same year, 31 million men had a DRE (Woodwell, 1999). Moreover, the annual number of newly diagnosed cases of prostate cancer in the United States is estimated at 179,000 (Landis et al., 1999). It is the second most commonly diagnosed cancer and second leading cause of cancer mortality in Canadian men. In 1997 prostate cancer accounted for 19,800 of newly diagnosed cancers in Canadian men (28%) (National Cancer Institute of Canada). It is estimated that 30% to 40% of all men over the age of forty-nine (49) have some cancerous prostate cells, yet only 20% to 25% of these men have a clinically significant form of prostate cancer (SpringNet—CE Connection, internet, www.springnet.com/ce/j803a.htm). Prostate cancer exhibits a wide variety of histological behaviour involving both endogenous and exogenous factors, i.e. socio-economic situations, diet, geography, hormonal imbalance, family history and genetic constitution (Konishi et al. 1997; Hayward et al. 1998). Although certain mtDNA alterations have been previously associated with prostate cancer, the need exists for further markers for the detection of prostate cancer.

Breast Cancer

[0007] Breast cancer is a cancer of the glandular breast tissue and is the fifth most common cause of cancer death. In 2005, breast cancer caused 502,000 deaths (7% of cancer deaths; almost 1% of all deaths) worldwide (World Health Organization Cancer Fact Sheet No. 297). Among women worldwide, breast cancer is the most common cancer and the most common cause of cancer death (World Health Organization Cancer Fact Sheet No. 297). Although certain mtDNA alterations have been previously associated with breast cancer, for example in Parrella et al. (Cancer Research: 61, 2001), the need exists for further markers for the detection of breast cancer.

[0008] This background information is provided for the purpose of making known information believed by the applicant to be of possible relevance to the present invention. No admission is necessarily intended, nor should be construed, that any of the preceding information constitutes prior art against the present invention.

SUMMARY OF THE INVENTION

[0009] The present invention pertains to mitochondrial DNA mutations for use in the detection of cancer. In accordance with an aspect of the present invention, there is provided a method of detecting a cancer in an individual comprising:

[0010] a) obtaining a biological sample from the individual;

[0011] b) extracting mitochondrial DNA (mtDNA) from the sample;

[0012] c) quantifying the amount of mtDNA in the sample having a deletion in the mtDNA sequence between about residue 12317 and about residue 16254 of the human mtDNA genome; and

[0013] d) comparing the amount of mtDNA in the sample having the deletion to at least one known reference value.

[0014] In accordance with another aspect of the present invention, there is provided a method of monitoring an individual for the development of a cancer comprising:

[0015] a) obtaining a biological sample;

[0016] b) extracting mitochondrial DNA (mtDNA) from the sample;

[0017] c) quantifying the amount of mtDNA in the sample having a deletion in the mtDNA sequence between about residue 12317 and about residue 16254 of the human mtDNA genome; and

[0018] d) repeating steps a) to c) over a duration of time;

[0019] wherein an increasing level of the deletion over the duration of time is indicative of cancer.

[0020] In accordance with another aspect of the present invention, there is provided a method of detecting a cancer in an individual comprising:

[0021] a) obtaining a biological sample from the individual;

[0022] b) extracting mitochondrial DNA (mtDNA) from the sample;

[0023] c) quantifying the amount of mtDNA in the sample having a sequence corresponding to the sequence as set forth in SEQ ID NO: 1 or SEQ ID NO: 2; and

[0024] d) comparing the amount of mtDNA in the sample corresponding to SEQ ID NO: 1 or SEQ ID NO: 2 to at least one known reference value.

[0025] In accordance with another aspect of the present invention, there is provided a diagnostic kit for carrying out the method of the invention comprising:

[0026] (a) material for collecting one or more biological samples; and

[0027] (b) suitable primers and reagents for detecting the mtDNA deletion.

BRIEF DESCRIPTION OF THE FIGURES

[0028] These and other features of the invention will become more apparent in the following detailed description in which reference is made to the appended drawings.

[0029] FIG. **1** is a graph showing cycle threshold as related to Example 1.

[0030] FIG. **2** shows a ROC curve illustrating the specificity and sensitivity of one embodiment of the present invention.

[0031] FIG. **3** is a graph showing cycle threshold as related to Example 2.

[0032] FIG. **4** shows a ROC curve illustrating the specificity and sensitivity of another embodiment of the present invention.

[0033] FIG. **5** is a schematic diagram showing the design and sequence of a primer useful for the detection of the 4 kb deletion.

[0034] FIG. **6** shows a ROC curve illustrating the specificity and sensitivity of another embodiment of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

[0035] The present invention provides methods of predicting, diagnosing and monitoring cancer. The methods comprise obtaining one or more biological samples, extracting mitochondrial DNA (mtDNA) from the samples, quantifying the amount of a mitochondrial mutation in the samples and comparing the quantity of the mutation in a sample with a reference value. In this regard, the methods provide a comprehensive tool for determining disease onset and for assessing the predisposition of an individual to cancer. The methods also allow for the monitoring of an individual's risk factors over time and/or for monitoring a patient's response to therapeutic agents and treatment regimes.

DEFINITIONS

[0036] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

[0037] As used herein, the term "about" refers to an understood variation from the stated value. It is to be understood that such a variation is always included in any given value provided herein, whether or not it is specifically referred to.

[0038] As defined herein, "biological sample" refers to a tissue or bodily fluid containing cells from which mtDNA can be obtained. For example, the biological sample can be derived from tissue such as breast or prostate tissue, or from blood, saliva, cerebral spinal fluid, sputa, urine, mucous, synovial fluid, peritoneal fluid, amniotic fluid and the like. The biological sample may be a surgical specimen or a biopsy specimen. The biological sample can be used either directly as obtained from the source or following a pre-treatment to modify the character of the sample. Thus, the biological sample can be pre-treated prior to use by, for example, preparing plasma or serum from blood, disrupting cells, preparing liquids from solid materials, diluting viscous fluids, filtering liquids, distilling liquids, concentrating liquids, inactivating interfering components, adding reagents, and the like.

[0039] As used herein, "cycle threshold" (C_T) is the point at which target amplification using real-time PCR rises above background, as indicated by a signal such as a fluorescence signal. The C_T is inversely related to the quantity of the sequence being investigated.

[0040] As used herein, "diagnostic" or "diagnosing" means using the presence or absence of a mutation or combination of mutations as a factor in disease diagnosis or management. The detection of the mutation(s) can be a step in the diagnosis of a disease.

[0041] As used herein, "deletion" means removal of a region of mtDNA from a contiguous sequence of mtDNA. Deletions can range in size from one base to thousands of bases or larger.

[0042] As used herein, "mitochondrial DNA" or "mtDNA" is DNA present in mitochondria.

[0043] As used herein, "mutation" encompasses any modification or change in mitochondrial DNA from the wild type sequence, including without limitation point mutations, transitions, insertions, transversions, translocations, deletions, inversions, duplications, recombinations or combinations thereof. The modification or change of the sequence can extend from a single base change to the addition or elimination of an entire DNA fragment.

[0044] As defined herein, "sensitivity" refers to the fraction of true positives (true positive rate) results obtained using the method of the present invention.

[0045] As defined herein, "specificity" refers to the fraction of false positives (false positive rate) results obtained using the method of the present invention.

[0046] The terms "therapy" and "treatment," as used interchangeably herein, refer to an intervention performed with

the intention of improving a subject's status. The improvement can be subjective or objective and is related to ameliorating the symptoms associated with, preventing the development of, or altering the pathology of a disease. Thus, the terms therapy and treatment are used in the broadest sense, and include the prevention (prophylaxis), moderation, reduction, and curing of a disease, at various stages. Preventing deterioration of a subject's status is also encompassed by the term. Subjects in need of therapy/treatment thus include those already having the disease, as well as those prone to, or at risk of developing, the disease, and those in whom the disease is to be prevented.

Assays for Predicting, Diagnosing and Monitoring Cancer

Assay for Detection of Mitochondrial Mutation

[0047] Mitochondrial DNA (mtDNA) dynamics are an important diagnostic tool.

[0048] Mutations in mtDNA are often preliminary indicators of developing disease and may act as biomarkers indicative of risk factors associated with disease onset. As discussed herein, measuring the level of mitochondrial DNA aberration in a biological sample can determine the presence of one or more cancers and identify the potential risk or predisposition of a patient to one or more cancers. Furthermore, measurement of mtDNA at regular intervals can provide health care professionals with a real-time, quantitative monitoring tool for measuring the progression of a patient over time and/or as an assessment for treatment recommendations in order to determine their effectiveness in preventing or treating cancer.

[0049] The present invention, therefore, provides methods for predicting, diagnosing or monitoring cancer, comprising obtaining one or more biological samples, extracting mitochondrial DNA (mtDNA) from the samples, and assaying the samples for mitochondrial mutation by: quantifying the amount of an mtDNA aberration in the sample and comparing the level of the aberration with a reference value. As would be understood by those of skill in the art, the reference value is based on whether the method seeks to predict, diagnose or monitor cancer. Accordingly, the reference value may relate to mtDNA data collected from one or more known non-cancerous biological samples, from one or more known cancerous biological samples, and/or from one or more biological samples taken over time. These reference values are used for comparison with the mtDNA data collected from the one or more biological samples wherein, for example, a similar or elevated amount of deletion in the biological sample compared to the reference sample is indicative of a predisposition to or the onset of cancer, or wherein an increasing level of the deletion over time is indicative of cancer onset.

[0050] In accordance with an aspect of the invention, the methods for predicting, monitoring and diagnosing cancer comprise an assay for detecting and quantifying one or more mitochondrial mutations. In accordance with one embodiment of the invention, the mutation is an mtDNA deletion. In accordance with another embodiment, the mutation is an mtDNA deletion of 3926 bp of mtDNA (referred to herein as "the 4 kb deletion" or "4 kb sequence"). In accordance with yet another embodiment, the mutation is an mtDNA deletion having the sequence as set forth in SEQ ID NO:1 or SEQ ID NO:2, there being no difference between SEQ ID NO: 1 and SEQ ID NO: 2 when in circular form.

[0051] The 4 kb deletion spans approximately nucleotides 12317 and 16254 of the human mtDNA genome. The human mtDNA genome is listed herein as SEQ ID NO:3 (Genbank accession no. AC_000021). The 4 kb deletion is characterized by direct flanking repeats 12 by in size, with the repeats located at positions 12317-12328 and 16243 to 16254. The repeat sequence is 5'-TGCAACTCCAAA-3'. Thus, in accordance with one embodiment of the invention, the mutation is an mtDNA deletion of between about residue 12317 and about residue 16254 of the human mtDNA genome.

[0052] The inventors have determined, as provided by way of example below, that this deletion is associated with cancer and in particular prostate and breast cancer. Therefore, such deletion provides an accurate biomarker and, therefore, a valuable tool for the detection, diagnosis, or monitoring of cancer in at least these tissues.

[0053] The deletion results in the creation of two deletion monomers, one of 4 kb in size (small sublimon) and one of approximately 12.5 kb in size (large sublimon). The occurrence of the deletion may be detected by either identifying the presence of the small sublimon or the large sublimon, the 4 kb or 12.5 kb sequence respectively.

[0054] Exemplary methods for assaying the mitochondrial mutation are provided in the Example section. Extraction of mtDNA from a sample may be undertaken using any suitable known method. MtDNA extraction is followed by amplification of all or a region of the mitochondrial genome, and may include sequencing of the mitochondrial genome, as is known in the art and described, for example, in Current Protocols in Molecular Biology (Ausubel et al., John Wiley & Sons, New York, 2007). Likewise, methods for detecting the presence of mutations in the mtDNA can be selected from suitable techniques known to those skilled in the art. For example, analyzing mtDNA can comprise sequencing the mtDNA, amplifying mtDNA by PCR, Southern, Northern, Western South-Western blot hybridizations, denaturing HPLC, hybridization to microarrays, biochips or gene chips, molecular marker analysis, biosensors, melting temperature profiling or a combination of any of the above.

[0055] Any suitable means to sequence mitochondrial DNA may be used. Preferably, mtDNA is amplified by PCR prior to sequencing. The method of PCR is well known in the art and may be performed as described in Mullis and Faloona, 1987, Methods Enzymol., 155: 335. PCR products can be sequenced directly or cloned into a vector which is then placed into a bacterial host. Examples of DNA sequencing methods are found in Brumley, R. L. Jr. and Smith, L. M., 1991, Rapid DNA sequencing by horizontal ultrathin gel electrophoresis, Nucleic Acids Res. 19:4121-4126 and Luckey, J. A., et al, 1993, High speed DNA sequencing by capillary gel electrophoresis, Methods Enzymol. 218: 154-172. The combined use of PCR and sequencing of mtDNA is described in Hopgood, R., et al, 1992, Strategies for automated sequencing of human mtDNA directly from PCR products, Biotechniques 13:82-92 and Tanaka, M. et al, 1996, Automated sequencing of mtDNA, Methods Enzymol. 264: 407-421.

[0056] Although real-time quantitative PCR methods, as described in the examples below, represent the preferred means for detecting and quantifying the presence or absence of the 4 kb deletion, other methods would be well known to an individual of skill in the art and could be utilized as indicated above. In addition, quantification of the deletion could be made using Bio-Rad's Bioplex™ System and Suspension

Array technology. Generally, the method requires amplification and quantification of sequences using any known methods.

[0057] The following primer sequences are examples of primers that may be used for the detection of the 4 kb deletion:

[0058] 4 forward (binds to bases 12313-12328/16255-16267 of the human mtDNA genome) 5'-TTGGTGCAACTCCAAAGCCACCCCTCACC-3' (SEQ ID NO: 4);

[0059] 4 reverse (binds to bases 16391-16409 of the human mtDNA genome) 5'-AGGATGGTGGTCAAGGGAC-3' (SEQ ID NO: 5).

[0060] In one embodiment of the present invention, a pair of amplification primers are used to amplify a target region indicative of the presence of the 4 kb deletion. In this embodiment, one of the pair of amplification primers overlaps a spliced region of mtDNA after deletion of the 4 kb sequence has occurred and the mtDNA has reformed as a circular mtDNA molecule (eg. a splice at a position between 12328 and 16255 of the mtDNA genome). Therefore, extension of the overlapping primer can only occur if the 4 kb section is deleted. FIG. **5** is a schematic diagram showing the design and sequence of the primer (ie. SEQ ID NO: 4).

[0061] In another embodiment of the present invention, a pair of amplification primers are used to amplify a target region associated with the deleted 4 kb sequence. The deleted 4 kb sequence, upon deletion, may reform as a circular mtDNA molecule. In this embodiment, one of the pair of amplification primers overlaps the rejoining site of the ends of the 4 kb sequence. Thus, an increase in the amount of the 4 kb molecule detected in a sample is indicative of cancer.

[0062] In still another embodiment of the present invention, the breakpoint of the deletion is unknown thereby resulting in two possibilities for primer location. In this embodiment, two separate forward primers may be designed to amplify the target region associated with the deleted 4 kb sequence. The following primer sequences are examples of those that may be used for the detection of the 4 kb deletion in this scenario:

Forward Primers:

[0063] Primer A (binds to bases 12313-12328/16255-16267 of the human mtDNA genome) 5'-TTGGTGCAACTCCAAAGCCACCCCTCACC-3' (SEQ ID NO: 4);

[0064] Primer B (binds to bases 12302-12316 of the human mtDNA genome) 5'-CCCAAAAATTTTGGTGCAACTCCAAAGCCAC-3' (SEQ ID NO: 6).

Reverse Primer:

[0065] Primer C (binds to bases 16391-16409 of the human mtDNA genome) 5'-AGGATGGTGGTCAAGGGAC-3' (SEQ ID NO: 5).

[0066] As would be understood by a person of skill in the art, the forward primers A or B can be used with reverse primer C to create PCR products that are useful in qPCR assays.

Biological Sample

[0067] The present invention provides for diagnostic tests which involve obtaining or collecting one or more biological samples. In the context of the present invention, "biological sample" refers to a tissue or bodily fluid containing cells from which mtDNA can be obtained. For example, the biological sample can be derived from tissue including, but not limited to, breast, prostate, nervous, muscle, heart, stomach, colon tissue and the like; or from blood, saliva, cerebral spinal fluid, sputa, urine, mucous, synovial fluid, peritoneal fluid, amniotic fluid and the like. The biological sample may be obtained from a cancerous or non-cancerous tissue and may be a surgical specimen or a biopsy specimen.

[0068] The biological sample can be used either directly as obtained from the source or following a pre-treatment to modify the character of the sample. Thus, the biological sample can be pre-treated prior to use by, for example, preparing plasma or serum from blood, disrupting cells, preparing liquids from solid materials, diluting viscous fluids, filtering liquids, distilling liquids, concentrating liquids, inactivating interfering components, adding reagents, and the like.

[0069] One skilled in the art will understand that more than one sample type may be assayed at a single time (i.e. for the detection of more than one cancer). Furthermore, where a course of collections are required, for example, for the monitoring of risk factors or cancer over time, a given sample may be diagnosed alone or together with other sample taken throughout the test period. In this regard, biological samples may be taken once only, or at regular intervals such as biweekly, monthly, semi-annually or annually.

[0070] One of skill will also appreciate that mitochondrial DNA targets are in much greater abundance (approximately 1000 fold greater) than nucleic acid targets and as such sample sizes comprising extremely low yields of nucleic acids would be suitable for use with the present invention.

Applications for Predicating, Diagnosing and Monitoring Cancer

Diagnosing and Monitoring Cancer

[0071] The prevalence of cancer in most tissue types and age groups necessitates the availability of a tool to not only detect the presence of cancer, but also to monitor the success and appropriateness of preventative measures and therapies being advised to prevent onset, progression and spread of the disease. Measuring the level of mitochondrial DNA deletions in one or more biological samples of an individual can provide initial diagnosis of risk factors, cancer and/or stages of the disease.

[0072] The system and method of the present invention, for example, may be used to detect cancer at an early stage, and before any histological abnormalities. Furthermore, sample testing at regular intervals such as biweekly, monthly, semi-annually or annually (or any other suitable interval) can provide health care professionals with a real-time, quantitative monitoring tool to compare against treatment recommendations to determine their effectiveness in preventing or treating the disease.

[0073] Turning now to the examples, in one embodiment the present invention may be used for detecting the presence of pre-neoplasia, neoplasia and progression towards potential malignancy of prostate cancer and breast cancer. In one aspect, the present invention involves the detection and quantification of the 4 kb mtDNA deletion for the detection, diagnosis, and/or monitoring of cancer. In this method, mtDNA is extracted from a biological sample (for example body tissue, or body fluids such as urine, prostate massage fluid). The extracted mtDNA is then tested in order to determine the levels (ie. quantity) of the 4 kb deletion in the sample. In tests conducted by the present inventors, the levels of the deletion were found to be elevated in samples obtained from subjects

with cancer when compared to samples obtained from subjects without cancer. Based on the information and data supplied below, the inventors have concluded that elevated levels of the 4 kb deletion in human mtDNA is indicative of cancer.

[0074] In another embodiment, samples of, for instance prostate tissue, prostate massage fluid, urine or breast tissue, are obtained from an individual and tested over a period of time (eg. years) in order to monitor the genesis or progression of cancer. Increasing levels of the 4 kb deletion over time could be indicative of the beginning or progression of cancer.

[0075] One of ordinary skill in the art will appreciate that analysing one or more biological samples from an individual for quantification of a mitochondrial DNA target provides a means for a health care worker to monitor the effectiveness of treatment regimes. One of ordinary skill will also appreciate the utility of mtDNA analysis for use by health care providers in identifying (and providing recommendations for) lifestyle habits, such as poor diet and exercise, or activities that cause over exposure of an individual to known carcinogens (eg. tobacco, pollutants).

[0076] Another aspect of the invention provides methods for confirming or refuting the results of a cancer biopsy test from a biopsy sample (eg. prostate or breast cancer), comprising: obtaining non-cancerous tissue from a biopsy sample; and detecting and quantifying the amount of the 4 kb mtDNA deletion in the non-diseased tissue.

Determining Genetic Predisposition to Cancer

[0077] In order to fully evaluate an individual's risk of one or more cancers it is imperative that health care providers are provided with as much information as possible to understand and communicate their patient's risk factors. The utilization of the present invention to determine the level of mtDNA aberration will not only prove helpful in assessing an individual's susceptibility to one or more cancers, it provides a valuable tool to identify patients with greater risk who are potentially in need of more aggressive monitoring and treatment measures.

[0078] In this regard, the various examples provided below illustrate a difference in the amount of mtDNA having the 4 kb deletion between samples obtained from subjects having cancer, and subjects without cancer. The amount of the 4 kb deletion was found to be higher in the samples obtained from subjects having cancer. This determination was made by comparing the amount of the 4 kb deletion in the samples from known cancer cells and/or known non-cancer cells.

[0079] As such, the inventors determined that screening of biological samples would prove useful in identifying an individual's predisposition to one or more cancers. Thus, in accordance with one embodiment of the present invention there is provided a method for screening individuals for cancer from one or more biological samples comprising: obtaining the one or more samples, and detecting and quantifying the level of the 4 kb mtDNA deletion in the samples. In a specific embodiment of the invention, there is provided a method for screening individuals for prostate or breast cancer from a body fluid or tissue sample comprising; obtaining the body fluid or tissue sample, and detecting and quantifying the level of the 4 kb mtDNA deletion in the body fluid or tissue sample.

[0080] Age related accumulation of the 4 kb mtDNA deletion may also predispose an individual to, for example, prostate cancer or breast cancer, which is prevalent in middle aged and older men, and middle aged and older women, respectively. Similarly, an accumulation of the 4 kb mtDNA deletion may be associated with a particular lifestyle based on an individual's diet, exercise habits, and exposure to known carcinogens. Thus, in accordance with one aspect of the invention, a method is provided wherein regular cancer screening may take place by monitoring over time the amount of the 4 kb deletion in one or more biological samples, non-limiting examples of which include breast and prostate tissues or body fluids such as prostate massage fluid, or urine.

Evaluation of Therapeutic Agents

[0081] The method of the present invention may also be used for screening potential therapeutic agents for use in cancer treatment or for monitoring the therapeutic effect of such agents. The method of the present invention may be used to measure various biomarkers associated with the cancers identified herein. The ability to assess the level of DNA damage in any biological sample at any time point provides the foundation for a unique and informative screening test for an individual's health and to assess the safety and efficacy of existing and new therapeutic agents and treatment regimes. Furthermore, by identifying the specific genetic changes underlying a subject's state of health, it may be readily determined whether and to what extent a patient will respond to a particular therapeutic agent or regime.

Kits

[0082] The present invention provides diagnostic/screening kits for use in a clinical environment. Such kits could not only include one or more sampling means, but other materials necessary for the identification of mtDNA mutations.

[0083] The kits can optionally include reagents required to conduct a diagnostic assay, such as buffers, salts, detection reagents, and the like. Other components, such as buffers and solutions for the isolation and/or treatment of a biological sample, may also be included in the kit. One or more of the components of the kit may be lyophilised and the kit may further comprise reagents suitable for the reconstitution of the lyophilised components.

[0084] Where appropriate, the kit may also contain reaction vessels, mixing vessels and other components that facilitate the preparation of the test sample. The kit may also optionally include instructions for use, which may be provided in paper form or in computer-readable form, such as a disc, CD, DVD or the like.

[0085] In one aspect of the invention there is provided a kit for diagnosing cancer comprising means for extraction of mtDNA, primers, reagents and instructions.

[0086] In another aspect of the invention there is provided a kit for diagnosing cancer, for example prostate or breast cancer, comprising means for extraction of mtDNA, primers having the nucleic acid sequences recited in SEQ ID NOs: 4 and 5, reagents and instructions.

[0087] In another aspect of the invention there is provided a kit for diagnosing cancer, for example prostate or breast cancer, comprising means for extraction of mtDNA, primers having the nucleic acid sequences recited in SEQ ID NOs: 6 and 5, reagents and instructions.

[0088] To gain a better understanding of the invention described herein, the following examples are set forth. It will be understood that these examples are intended to describe

illustrative embodiments of the invention and are not intended to limit the scope of the invention in any way.

EXAMPLES

Example 1

Association of Prostate Cancer with 4 kb Deletion in Human mtDNA

[0089] Urine samples were collected from five patients who had been diagnosed with prostate cancer and five who had a needle biopsy procedure which was unable to detect prostate malignancy. These samples were collected following a digital rectal exam (DRE) to facilitate the collection of prostate cells.

[0090] Upon receipt of the samples a 5 ml aliquot was removed and then 2 mls were centrifuged at 14,000×g to form a pellet. The supernatant was removed and discarded.

[0091] Pellets were resuspended in 200 ul phosphate buffered saline solution. Both the resuspended pellet and the whole urine sample were subjected to a DNA extraction procedure using the QiaAMP DNA Mini Kit (Qiagen P/N 51304) according to the manufacturer's directions. The resulting DNA extracts were then quantified using a NanoDrop ND-1000 Spectrophotometer and normalized to a concentration of 0.1 ng/ul.

[0092] Samples were analyzed by quantitative real-time PCR with the 4 kb deletion specific primers according to the following:

[0093] 1× iQ SYBR Green Supermix (Bio-Rad product no. 170-8880)

[0094] 100 nmol forward primer (5'-TTGGTGCAACTCCAAAGCCACCCCTCACC-3') (SEQ ID NO: 4)

[0095] 100 nmol reverse primer (5'-AGGATGGTGGTCAAGGGAC-3') (SEQ ID NO: 5)

[0096] 1 ng template DNA in a 25 ul reaction

[0097] Reactions were cycled on an Opticon 2 DNA Engine (Bio-Rad Canada) according to the following protocol:

[0098] 1. 95° C. for 3 minutes

[0099] 2. 95° C. for 30 seconds

[0100] 3. 69° C. for 30 seconds

[0101] 4. 72° C. for 30 seconds

[0102] 5. Plate Read

[0103] 6. Repeat steps 2-5 44 times

[0104] 7. 72° C. for 10 minutes

[0105] 8. Melting Curve from 50° C. to 105° C., read every 1° C., hold for 3 seconds

[0106] 9. 10° C. Hold

Results

[0107] Results from the urine pellet did not yield significant differences in the mean cycle threshold observed or a useful cutoff point. However, the results from the whole urine sample did yield significant differences as provided below.

[0108] Tables 1 and 2, and FIG. **1** show the difference in the mean C_T scores for urine samples from subjects having prostate malignant tissue and benign tissue at the 0.04 significance level.

TABLE 1

Mean Values for C_T scores: Urine Samples

	N	Mean	Std. Deviation	Std. Error Mean
Benign	7	38.0357	3.40974	1.288876
Malignant	7	31.9300	6.12583	2.31534

TABLE 2

Significance Test for Mean C_T scores
Independent Samples Test

	Levene's Test for Equality of Variances		Test for Equality Means						
CTt40					Sig. (2-	Mean	Std. Error	95% Confidence Interval of the Difference	
fluid	F	Sig.	t	df	tailed)	Diff.	Diff.	Lower	Upper
Equal variances assumed	1.707	.216	2304	12	.040	610571	264985	.33218	11.87925
Equal variances not assumed			2304	9392	.046	610571	264985	.14927	12.06215

[0109] Tables 3 and 4, and FIG. **2** illustrate that when using a cut-off cycle threshold of 36.255 the sensitivity of the assay for prostate cancer is 86% and the specificity is 86%.

[0110] FIG. **2** is a Receiver Operating Characteristic (ROC) curve illustrating the specificity and sensitivity of the 4 kb mtDNA deletion as a marker for prostate cancer when testing urine. These results were obtained using a cutoff C_T of 36.255. The sensitivity of the marker at this C_T is 86%, while the specificity is 86%.

[0111] The determination of the cutoff C_T of 36.255 is shown in Table 3. The results listed in Table 3 show that a cutoff C_T of 36.255 provided the highest sensitivity and specificity.

[0112] The accuracy of the test depends on how well the test separates the group being tested into those with and without the prostate cancer. Accuracy is measured by the area under the ROC curve. Table 4 shows the calculation of the area under the curve for the present example.

TABLE 3

Determination of Specificity and Sensitivity

Positive if ≦[a]	Sensitivity	1 − specificity
19.86	.000	.000
24.87	.143	.000
29.48	.286	.000
30.54	.429	.000
32.235	.429	.143
33.77	.571	.143
35.11	.714	.143
36.255	.857	.143
37.415	.857	.286
39.23	.857	.429
39.995	1.000	.429
40.21	1.000	.857
41.42	1.000	1.000

[a]the smallest cutoff value is the minimum observed test value minus 1 and the largest cutoff value is the maximum observed test value plus 1. All the other cutoff values are the averages of two consecutive ordered observed test values.

TABLE 4

Results Showing Area Under the ROC Curve

			Asymptotic 95% Confidence Interval	
Area	Std. Error[a]	Asymptotic Sig.[b]	Lower bound	Upper bound
.878	.096	.018	.689	1.066

Notes:
[a]under the non-parametric assumption
[b]null hypothesis: true area = 0.5

Example 2

Association of Breast Cancer with 4 kb Deletion in Human mtDNA

[0113] Twenty breast tissue samples were collected, ten of which were malignant and ten of which had benign breast disease or no abnormalities. These samples were formalin-fixed paraffin embedded and 20 micron sections of each were cut into individual sample tubes for extraction according to the manufacturer's protocol for the QiaAMP DNA Mini Kit (Qiagen P/N 51304). DNA was then quantified using a Nano-drop ND-1000 and normalized to a concentration of 2 ng/ul.

[0114] Samples were then assayed for the levels of the 4 kb deletion by quantitative real-time PCR using the following protocol:

[0115] X iQ SYBR Green Supermix (Bio-Rad product no. 170-8880)

[0116] 175 nmol forward primer (5'-TTGGTGCAACTC-CAAAGCCACCCCTCACC-3') (SEQ ID NO: 4)

[0117] 175 nmol reverse primer (5'-AGGATGGTGGT-CAAGGGAC-3') (SEQ ID NO: 5)

[0118] 20 ng template DNA in a 25 ul reaction

[0119] Reactions were cycled on an Opticon 2 DNA Engine (Bio-Rad Canada) according to the following protocol:

[0120] 1.95° C. for 3 minutes

[0121] 2. 95° C. for 30 seconds

[0122] 3. 70° C. for 30 seconds

[0123] 4. 72° C. for 30 seconds

[0124] 5. Plate Read

[0125] 6. Repeat steps 2-5 44 times

[0126] 7. 72° C. for 10 minutes

[0127] 8. Melting Curve from 50° C. to 105° C., read every 1° C., hold for 3 seconds

[0128] 9. 10° C. Hold

[0129] Tables 5 and 6, and FIG. **3** show the difference in the mean C_T scores for breast tissue samples from subjects having malignant breast tissue and benign breast tissue at the 0.065 level.

TABLE 5

Mean Values for C_T scores: Breast Tissue Samples

Group	N	Mean	Std. Dev.	Std. Error Mean
Normal	9	21.5278	2.71939	.90646
Malignant	9	18.9089	2.89126	.96375

TABLE 6

Significance Test for Mean C_T scores

			Test for Equality Means						
	Levene's Test for Equality of Variances				Sig. (2-	Mean	Std. Error	95% Confidence Interval of the Difference	
CTt40 fluid	F	Sig.	t	df	tailed)	Diff.	Diff.	Lower	Upper
Equal variances assumed	.007	.934	1.979	16	.065	2.61889	1.32306	−.18588	5.42366
Equal variances not assumed			1.979	15.94	.065	2.61889	1.32306	−.18674	5.42452

[0130] Tables 7 and 8, and FIG. **4** illustrate that when using a cut-off cycle threshold of 19.845 the sensitivity of the assay for breast cancer is 78% and the specificity is 78%.

[0131] FIG. **4** is an ROC curve illustrating the specificity and sensitivity of the 4 kb mtDNA deletion as a marker for breast cancer when testing breast tissue. These results were obtained using a cutoff C_T of 19.845. The sensitivity of the marker at this C_T is 78%, while the specificity is 78%.

[0132] The determination of the cutoff C_T of 19.845 is shown in Table 7. The results listed in Table 7 show that a cutoff C_T of 19.845 provided the highest sensitivity and specificity.

[0133] The accuracy of the test depends on how well the test separates the group being tested into those with and without the breast cancer. Accuracy is measured by the area under the ROC curve. Table 8 shows the calculation of the area under the curve for the present example.

TABLE 7

Determination of Specificity and Sensitivity

Positive if ≦[a]	Sensitivity	1 – specificity
15.28	.000	.000
16.305	.111	.000
16.69	.222	.000
17.075	.333	.000
17.4	.444	.000
17.71	.556	.000
18.0	.556	.111
18.835	.556	.222
19.415	.667	.222
19.845	.778	.222
20.475	.778	.333
10.79	.778	.444
21.38	.778	.556
22.005	.778	.667
23.145	.889	.667
24.19	.889	.778
24.49	.889	.889
25.21	1.00	.889
26.66	1.00	1.00

[a]the smallest cutoff value is the minimum observed test value minus 1 and the largest cutoff value is the maximum observed test value plus 1. All the other cutoff values are the averages of two consecutive ordered observed test values.

TABLE 8

Results Showing Area Under the ROC Curve

			Asymptotic 95% Confidence Interval	
Area	Std. Error[a]	Asymptotic Sig.[b]	Lower bound	Upper bound
.778	.117	.047	.548	1.008

Example 3

Association of Prostate Cancer with 4 kb Deletion in Human mtDNA Using Needle Biopsy Samples

[0134] Prostate needle biopsy specimens were obtained from 19 individuals, 9 without prostate cancer and 10 with prostate cancer. Needle biopsy tissues were formalin-fixed paraffin embedded (FFPE) as is standard in the clinical diagnostic setting. 10 micron sections of each biopsy were deposited directly into centrifuge tubes and the DNA was extracted using the QiaAMP DNA Mini Kit (Qiagen, p/n 51306). DNA extracts were quantified by absorbance at 260 nm using a NanoDrop ND-1000 Spectrophotometer. Yields ranged from 347 ng to 750 ng. These samples were diluted to 2 ng/ul and amplification reactions setup according to Table 9 and the following:

TABLE 9

Reagents and Concentrations for Amplification Reaction

Reagent	Final Concentration
iQ SYBR Green Supermix (Bio-Rad Laboratories, p/n 170-8882)	1 X
Forward Primer 12303-12316/16243-16259F 5'- CCCAAAAATTTGGTGCAACTCCAAAGCCAC -3' (SEQ ID NO: 6)	175 nmol
Reverse Primer 16410R 5'- AGGATGGTGGTCAAGGGAC -3' (SEQ ID NO: 5)	175 nmol
DNA extract	0.8 ng/ul

[0135] Nuclease-free water was added to a final reaction volume of 25 ul. Amplifications were carried out on a DNA Engine Chromo4 Real Time PCR Instrument (Bio-Rad Laboratories) according the following cycling conditions:

[0136] 1) 95° C. for 3 minutes

[0137] 2) Followed by 45 cycles of

[0138] 3) 95° C. for 30 seconds

[0139] 4) 69° C. for 30 seconds

[0140] 5) 72° C. for 30 seconds

[0141] 6) Plate Read

Then

[0142] 7) 72° C. for 10 minutes

[0143] 8) Melting Curve 50° C.-105° C. reading every 1° C., hold for 3 seconds

[0144] 9) 4° C. Hold

[0145] Results, shown in Table 10, demonstrate that those individuals with prostate cancer have a lower C_T value and therefore higher levels of the 4 kb deletion in prostate tissue than do those without prostate cancer. Patients with prostate cancer have an average C_T value of 30.7 while the patients without prostate cancer have an average C_T value of 36.4. This difference of 5.7 C_T corresponds to nearly 100 fold greater 4 kb deletion levels in the group with prostate malignancy than in the group without.

TABLE 10

Patient Diagnosis and Associated C_T Score

Patient Number and Diagnosis	C(t)
CUG 1301 Malignant	25.7
CUG 1268 Malignant	27.7
CUG RN 345 Normal	28.3
CUG 1272 Malignant	28.8
CUG 1375 Malignant	29.1
CUG 1259 Malignant	29.1
CUG 1381 Malignant	30.2
CUG RN 82 Normal	30.5
CUG 1372 Malignant	30.9

TABLE 10-continued

Patient Diagnosis and Associated C_T Score

Patient Number and Diagnosis	C(t)
CUG 1085 C T1 Normal	31.5
CUG 1317 Malignant	31.7
CUG 1377 F Normal	33.6
CUG 1365 B Normal	34.6
CUG 1370 Malignant	35.9
CUG RN 405 Normal	37.5
CUG 1366 Malignant	37.9
CUG RN 701 Normal	41.7
CUG RN 420 Normal	45
CUG RN 373 Normal	45

[0146] Tables 11 and 12 show the difference in the mean C_T scores for prostate tissue samples from subjects having normal and malignant prostate tissue.

TABLE 11

Mean Values for C_T Score: Prostate Needle Biopsy Tissue

Group	N	Mean	Std. Dev.	Std. Error Mean
Normal	9	36.4111	6.25229	2.08410
Malignant	10	30.7	3.69534	1.16857

TABLE 12

Significance Test for C_T Scores

	Levene's Test for Equality of Variances		Test for Equality Means						
					Sig. (2-	Mean	Std. Error	95% Confidence Interval of the Difference	
CTt40 fluid	F	Sig.	t	df	tailed)	Diff.	Diff.	Lower	Upper
Equal variances assumed	4.426	.051	2.455	17	.025	5.71111	2.32589	.80391	10.61831
Equal variances not assumed			2.390	12.705	.033	5.71111	2.38935	.53701	10.88522

[0147] Table 13 and FIG. **6** illustrate that when using a cutoff of C_T 32.65 the sensitivity and specificity of correctly diagnosing these patients is 80% and 67% respectively.

TABLE 13

Determination of Specificity and Sensitivity

Positive if $\geq^a$	Sensitivity	1 – specificity
24.7	.000	.000
26.7	.100	.000
28.0	.200	.000
28.55	.200	.111
28.95	.300	.111
29.65	.500	.111
30.35	.600	.111
30.7	.600	.222

TABLE 13-continued

Determination of Specificity and Sensitivity

Positive if $\geq^a$	Sensitivity	1 – specificity
31.2	.700	.222
31.6	.700	.333
32.65	.800	.333
34.1	.800	.444
32.25	.800	.556
36.7	.900	.556
37.7	.900	.667
39.8	1.000	.667
43.35	1.000	.778
46.0	1.000	1.000

[0148] Although the invention has been described with reference to certain specific embodiments, various modifications thereof will be apparent to those skilled in the art without departing from the spirit and scope of the invention as outlined in the claims appended hereto. All such modifications as would be apparent to one skilled in the art are intended to be included within the scope of the following claims. All documents recited in the present application are incorporated herein by reference.

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1. A method of detecting a cancer in an individual comprising:
a) obtaining a biological sample from the individual;
b) extracting mitochondrial DNA (mtDNA) from the sample;
c) quantifying the amount of mtDNA in the sample having a deletion in the mtDNA sequence between about residue 12317 and about residue 16254 of the human mtDNA genome; and
d) comparing the amount of mtDNA in the sample having the deletion to at least one known reference value.

2. The method of claim **1** wherein the deletion has a sequence as set forth in SEQ ID NO: 1 or SEQ ID NO: 2.

3. The method of claim **1** wherein the at least one known reference value is the amount of the deletion in a reference sample of mtDNA from known non-cancerous tissue or body fluid.

4. The method of claim **3** wherein an elevated amount of the deletion in the biological sample compared to the reference sample is indicative of cancer.

5. The method of claim **4** further comprising the step of comparing the amount of mtDNA in the sample having the deletion to the amount of the deletion in a reference sample of mtDNA from known cancerous tissue or body fluid.

6. The method of claim **1** wherein the at least one known reference value is the amount of the deletion in a reference sample of mtDNA from known cancerous tissue or body fluid.

7. The method of claim **6** wherein a similar level of the deletion in the biological sample compared to the reference sample is indicative of cancer.

8. The method of claim **7** further comprising the step of comparing the amount of mtDNA in the sample having the deletion to the amount of the deletion in a reference sample of mtDNA from known non-cancerous tissue or body fluid.

9. The method of claim **1** wherein the step of quantifying is conducted using real-time PCR.

10. The method of claim **1** wherein the quantifying of the deletion includes first amplifying a target region of mtDNA that is indicative of the deletion, and quantifying the amount of the amplified target region.

11. The method of claim **10** wherein a PCR primer having a sequence corresponding to SEQ ID NO: 4 is used as part of a pair of amplification primers for amplifying the target region.

12. The method of claim **1** wherein the cancer is prostate cancer.

13. The method of claim **1** wherein the cancer is breast cancer.

14. The method of claim **1** wherein the biological sample is a body tissue or body fluid.

15. The method of claim **14** wherein the biological sample is selected from the group consisting of breast tissue, prostate tissue and urine.

16. The method of claim **10** wherein the reference value is a cycle threshold.

17. A method of monitoring an individual for the development of a cancer comprising:
a) obtaining a biological sample;
b) extracting mitochondrial DNA (mtDNA) from the sample;
c) quantifying the amount of mtDNA in the sample having a deletion in the mtDNA sequence between about residue 12317 and about residue 16254 of the human mtDNA genome; and
d) repeating steps a) to c) over a duration of time;
wherein an increasing level of the deletion over the duration of time is indicative of cancer.

18. The method of claim **17** wherein the deletion has a sequence as set forth in SEQ ID NO: 1 or SEQ ID NO: 2.

19. The method of claim **17** further comprising at least one step selected from the group consisting of: (a) comparing the amount of mtDNA in the sample having the deletion to the amount of the deletion in a reference sample of mtDNA from known non-cancerous tissue or body fluid; and (b) comparing the amount of mtDNA in the sample having the deletion to the amount of the deletion in a reference sample of mtDNA from known cancerous tissue or body fluid.

20. The method of claim **17** wherein the quantifying of the deletion includes amplifying a target region of mtDNA that is indicative of the deletion, and quantifying the amount of the amplified target region.

21. The method of claim **20** wherein the step of quantifying is conducted using real-time PCR.

22. The method of claim **20** wherein a PCR primer having a sequence corresponding to SEQ ID NO: 2 is used as part of a pair of amplification primers for amplifying the target region.

23. The method of claim **17** wherein the cancer is prostate cancer.

24. The method of claim **17** wherein the cancer is breast cancer.

25. The method of claim **17** wherein the biological sample is a body tissue or body fluid.

26. The method of claim **25** wherein the biological sample is selected from the group consisting of breast tissue, prostate tissue and urine.

27. The method of claim **10** wherein the amplifying of the target region is conducted using a pair of amplification primers, one of the pair of amplification primers overlapping a splice joining regions on opposite ends of the deletion.

28. A method of detecting a cancer in an individual comprising:

a) obtaining a biological sample from the individual;

b) extracting mitochondrial DNA (mtDNA) from the sample;

c) quantifying the amount of mtDNA in the sample having a deletion corresponding to the sequence as set forth in SEQ ID NO: 1 or SEQ ID NO: 2; and

d) comparing the amount of mtDNA in the sample corresponding to SEQ ID NO: 1 or SEQ ID NO: 2 to at least one known reference value.

29. The method of claim **28** wherein the at least one known reference value is the amount of the sequence corresponding to SEQ ID NO: 1 or SEQ ID NO: 2 in a reference sample of mtDNA from known non-cancerous tissue or body fluid.

30. The method of claim **28** wherein the at least one known reference value is the amount of the sequence corresponding to SEQ ID NO: 1 or SEQ ID NO: 2 in a reference sample of mtDNA from known cancerous tissue or body fluid.

31. The method of claim **28** wherein the step of quantifying is conducted using real-time PCR.

32. The method of claim **31** wherein the quantifying of the deletion includes first amplifying a target region of mtDNA that is indicative of the deletion, and quantifying the amount of the amplified target region.

33. The method of claim **32** wherein one of a pair of PCR primers used in the amplifying of the target region overlaps a rejoining site of the sequence corresponding to SEQ ID NO: 1 or SEQ ID NO: 2, after the sequence has re-circularized.

34. The method of claim **28** wherein the cancer is prostate cancer.

35. The method of claim **28** wherein the cancer is breast cancer.

36. The method of claim **28** wherein the biological sample is a body tissue or body fluid.

37. The method of claim **36** wherein the biological sample is selected from the group consisting of breast tissue, prostate tissue and urine.

38. The method of claim **32** wherein the reference value is a cycle threshold.

39. A diagnostic kit for carrying out the method of claim **1** comprising:

(a) material for collecting one or more biological samples; and

(b) suitable primers and reagents for detecting the mtDNA deletion.

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